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Lack of translocation of protein kinase C from the cytosol to the membranes in vasopressin-stimulated hepatocytes

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The ability of Ca^{2+}-mobilizing hormones to promote changes in the subcellular distribution of protein kinase C (PKC) was studied in isolated hepatocytes. In recently isolated cells the distribution of PKC between the soluble and particulate fractions was 47 and 53% respectively. Exposure of the hepatocytes to 100 nM-vasopressin produced an increased phosphoinositide turnover, as reflected by the changes in the concentrations of inositol trisphosphate and Ca^{2+}, and in glycogen phosphorylase *a* activity. However, the distribution of both PKC activity and [^{3}H]phorbol dibutyrate binding between the cytosol and the membranes remained unchanged under these conditions. To determine the threshold values of the concentrations of Ca^{2+} and diacylglycerol required to produce a redistribution of PKC, the hepatocytes were treated with the Ca^{2+} ionophore ionomycin, and with permeant diacylglycerol derivatives. Hepatocytes incubated in the presence of 100 nM-vasopressin required concentrations of Ca^{2+} 2.5 times those produced physiologically by the hormone to produce translocation of PKC from the cytosol to the membranes. These studies suggest that, at least in hepatocytes, activation of PKC in response to Ca^{2+}-mobilizing hormones involves only the pre-existent membrane-bound enzyme without affecting the soluble enzyme.

INTRODUCTION

The occupancy of [arginine]vasopressin (VP) receptors of the hepatocyte plasma membrane elicits the generation of intracellular signals through the participation of a guanine-nucleotide-binding protein (G-protein) that acts as an amplifying and regulatory component of the extracellular signal-transduction system [1–3]. This G-protein, in turn, activates a phosphatidylinositol-specific phospholipase C which leads to a rapid increase in the concentration of diacylglycerol (DAG) and inositol phosphates [4–7], the latter being responsible for the transient rise in the free cytosolic Ca^{2+} concentration [8,9].

One of the rapid responses to the increased phosphoinositide turnover is the activation of protein kinase C (PKC) by the synergistic action of DAG and Ca^{2+} [10,11]. This kinase participates in a wide type of biological responses, among them being the control of several steps of the intermediary metabolism and phosphorylation of membrane proteins [12–15].

PKC is distributed between the cytosol and particulate fractions of the cells. Only the membrane-bound enzyme seems to participate in the mediation of biological responses elicited through phosphoinositide turnover [16,17]. Stimuli-dependent translocation of the enzyme to the membranes should therefore be expected for hormones using this pathway. However, in metabolically active tissues such as brain or liver, a high percentage of the enzyme is continuously present in particulate fractions, whereas in quiescent and resting cells most of the activity is found in the cytosol [12,18,19]. The physiological role of the membrane-bound enzyme in the absence of added extracellular stimuli is not clear. In the brain, it has been proposed that this particulate enzyme may participate in the mechanism of memory [20].

In this context we investigated the subcellular distribution of PKC in hepatocytes exposed to VP. The aim of the present work was to determine whether the rises in the concentrations of DAG and Ca^{2+} elicited by VP influence the subcellular compartmentation of the enzyme. The measurements of the changes in the intracellular concentrations of Ca^{2+}, InsP_3 and the activity of glycogen phosphorylase, an enzyme very sensitive to changes in Ca^{2+} concentration, may serve as controls for the function of the VP signalling pathway in the hepatocytes. In this report we show that exposure of hepatocytes to saturating concentrations of VP did not produce changes in the distribution of PKC. In addition, we provide quantitative information on the amounts of the second messengers (mainly Ca^{2+}) required to mobilize PKC from the cytosol to the membranes.

MATERIALS AND METHODS

Chemicals

[γ-^{32}P]ATP (3000 Ci/mol) and [^{3}H]phorbol 12,13-dibutyrate (PDBu, 20 Ci/mmol) were from New England Nuclear (Boston, MA, U.S.A.). InsP_3 assay system was from Amersham International (Amersham, Bucks., U.K.). Fura-2 probes were from Molecular Probes (Eugene, OR, U.S.A.). Ionomycin was from Calbiochem, VP, histone H1, phosphatidylserine, dioctanoylglycerol, 1-oleoyl-2-acetyl-*rac*-glycerol (OAG) and phorbols were from Sigma (St. Louis, MO, U.S.A.). Nonidet P-40 was from USB (Cleveland, OH, U.S.A.). Other chemicals were from Merck or Boehringer.

Isolation and incubation of hepatocytes

Hepatocytes were isolated from male Wistar rat livers by digestion with collagenase [21]. The hepatocytes were filtered through a 100 μm nylon mesh, and washed three times with Krebs–Ringer bicarbonate buffer saturated with O_2/CO_2 (19:1) by centrifugation at 50 *g* for 2 min in a refrigerated centrifuge (Minifuge T, Haereus). This procedure gave a cell preparation virtually free of membrane fragments, as assessed by microscopic observation. The hepatocyte suspension contained 100–150 mg of cells/ml. Only preparations with cell viability higher than 90%, determined by Trypan Blue exclusion, were used.

The incubations were stopped by centrifugation for 10 s in an

Abbreviations used: DAG, diacylglycerol; OAG, 1-oleoyl-2-acetyl-*rac*-glycerol; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C (EC 2.7.1.37); VP [arginine]vasopressin.

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Eppendorf centrifuge. Samples for the measurement of enzymic activities were immediately frozen in liquid N_2 (0.3 and 1.5 ml samples for glycogen phosphorylase and PKC respectively). For determination of InsP_3, the cells were treated with 1 vol of ice-cold 0.2 M-$HClO_4$ and stored at 4 °C.

Measurement of metabolites

The absolute mass of InsP_3 was measured by following the recommendations of the supplier. Appropriate internal standards were used to determine the accuracy of the assay [22,23].

To measure the intracellular Ca^{2+} concentration, the hepatocytes were transferred at 20 mg of cells/ml to a modified Krebs–Ringer buffer in which the bicarbonate was replaced by 20 mM-Hepes, pH 7.4, to decrease the noise caused by the appearance of CO_2 bubbles, and filtered through a 100 μm nylon mesh. The cells were loaded at 37 °C with fura-2 AM for 10 min (final concn. 5 μM) with continuous shaking (40 cycles/min). After this incubation, 3 ml samples of the hepatocyte suspension were washed twice by centrifugation at 100 *g* for 2 min, resuspended in warm medium and placed in a spectrofluorimetric cuvette provided with continuous magnetic stirring. The fluorescence was recorded in a Perkin–Elmer L50 spectrofluorimeter. The maximal fluorescence was determined at the end of the assay by adding 10 μl of 10% (w/v) SDS. The minimal fluorescence was obtained by adding 30 μl of 0.2 M-EGTA (pH 9.0) [24].

Measurement of enzyme activities

Glycogen phosphorylase *a* activity was measured essentially as described by Stalmans & Hers [25], by monitoring the phosphate release from glucose 1-phosphate (50 mM) in the presence of 100 mM-NaF, 1% glycogen and 0.5 mM-caffeine at pH 6.1.

To measure PKC activity, the hepatocyte pellets (200–300 mg of cells) were homogenized in 2 ml of ice-cold 0.25 M-sucrose containing 10 mM-β-mercaptoethanol, 1 mM-EGTA, 1 mM-EDTA, 1 mM-phenylmethanesulphonyl fluoride, 10 μg of leupeptin/ml and 20 mM-Hepes, pH 7.4 (buffer A). The homogenate was centrifuged at 105000 *g* for 30 min, yielding a soluble and a particulate fraction (pellet). To extract the membrane-bound enzyme, the pellet was resuspended in 2 ml of buffer A supplemented with 0.1% Nonidet P-40, followed by centrifugation (105000 *g* for 20 min). The soluble and membrane-bound extracted enzymes were partially purified by chromatography on DEAE-cellulose (DE52) after elution with 150 mM-NaCl to remove the inhibitors of the enzyme present in the homogenate [26]. The activity of PKC was monitored by the phosphorylation of histone H1 in the absence and presence of Ca^{2+}, phosphatidylserine and DAG as previously described [27,28]. One unit of PKC was defined as incorporating 1 nmol of phosphate into histone H1/min.

[^{3}H]PDBu-binding assay

Binding of [^{3}H]PDBu was measured in the soluble and particulate fractions of 1:4 (w/v) homogenates of hepatocytes [29,30]. For the cytosolic activity, the supernatants were desalted by gel filtration on Sephadex G-25 (medium grade) equilibrated with 100 mM-KCl/5 mM-$MgCl_2$/0.05 mM-EGTA/10 mM-β-mercaptoethanol/leupeptin (10 μg/ml)/20 mM-Hepes, pH 7.5. The particulate fractions were resuspended in the same medium. The PDBu binding was performed by incubating for 10 min at 30 °C, in 150 μl, a mixture containing 100 μl of sample, 100 μM-Ca^{2+} and [^{3}H]PDBu in the absence or in the presence of 2 μM non-radioactive PDBu. The assay for the cytosol contained 100 μg of sonicated phosphatidylserine/ml. Equilibrium binding was reached after 1–2 min of incubation. To calculate the specific binding, the binding in the presence of an excess of unlabelled PDBu (2 μM) was subtracted from the corresponding assay [27].

Translocation of PKC *in vitro*

This was analysed by incubating plasma membranes [31] with soluble PKC purified by DE52 chromatography. Samples of incubation mixtures (200 μl) were collected at various times, centrifuged at 105000 *g* for 30 min, and the activity of PKC was measured in the soluble and membrane-bound fractions after extraction with buffer A in the presence of 0.1% Nonidet P-40. All reagents used for these experiments were prepared in Chelex-treated water (Na^+ form).

Protein was determined as described by Bradford [32], with bovine serum albumin as standard.

RESULTS

To analyse the effect of VP on the subcellular distribution of PKC, the hepatocytes were incubated with the agonist, and samples were periodically collected to determine the activities of glycogen phosphorylase and of PKC in the soluble and particulate fractions, and the content of InsP_3. Fig. 1 shows the evolution of these parameters in hepatocytes incubated with 100 nM-VP. The stimulation of glycogen phosphorylase activity and the rise in the concentration of InsP_3 agree with previously reported results [22,23,33,34]. Despite the rise in the phosphoinositide turnover elicited by VP, the distribution of PKC between soluble and particulate fractions remained virtually unchanged, at least during the 12 min period of observation.

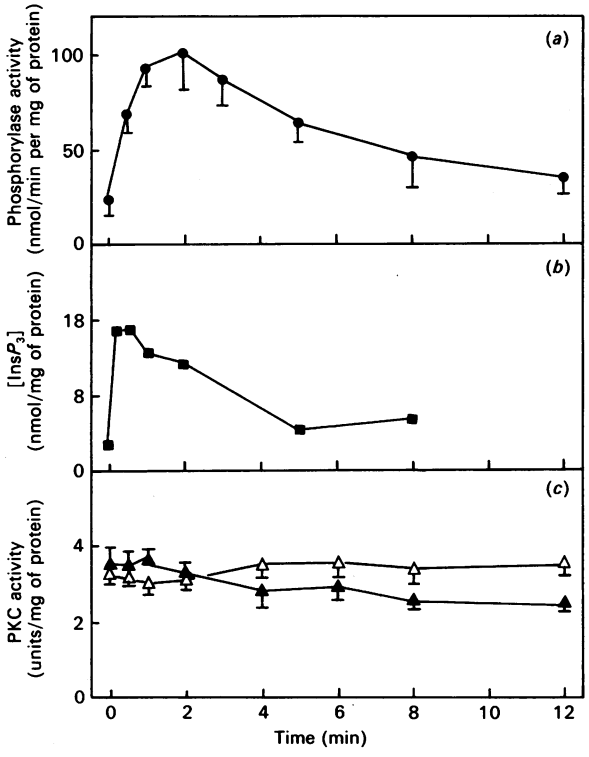

Fig. 1. Time-dependent changes in the activity of glycogen phosphorylase *a* (*a*), InsP_3 concentration (*b*) and PKC distribution (*c*) in isolated hepatocytes incubated with 100 nM-VP

At the indicated times, samples of the cell incubations were collected to measure these parameters. The distribution of PKC between the soluble (△) and particulate (▲) fractions was measured after partial purification of the enzyme by DE52 chromatography. Results are means ± S.E.M. for three different determinations, except for the measurement of InsP_3 ($n = 2$).

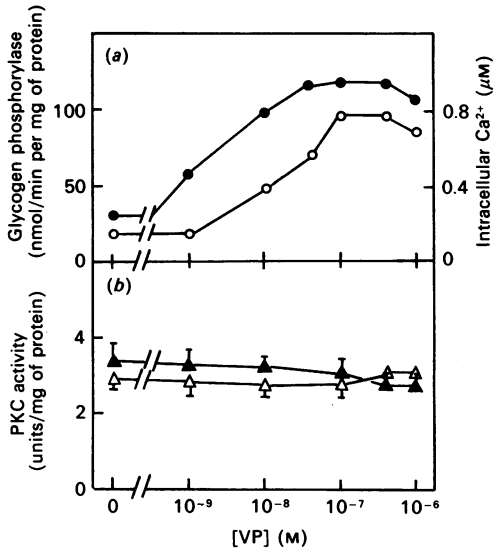

Fig. 2. Dose-dependent effect of VP on the intracellular Ca^{2+} concentration and glycogen phosphorylase *a* activity (*a*) and PKC distribution (*b*) in isolated hepatocytes

The cells were incubated for 2 min with the hormone, and samples were collected for the measurement of glycogen phosphorylase (●), the intracellular Ca^{2+} concentration (○) (*a*) and the soluble (△) and particulate (▲) activity of PKC (*b*). The changes in Ca^{2+} were monitored by the fluorescence of fura-2-loaded hepatocytes. The maximal increase in the concentration of Ca^{2+} is shown. Results are means ± S.E.M. of three independent experiments.

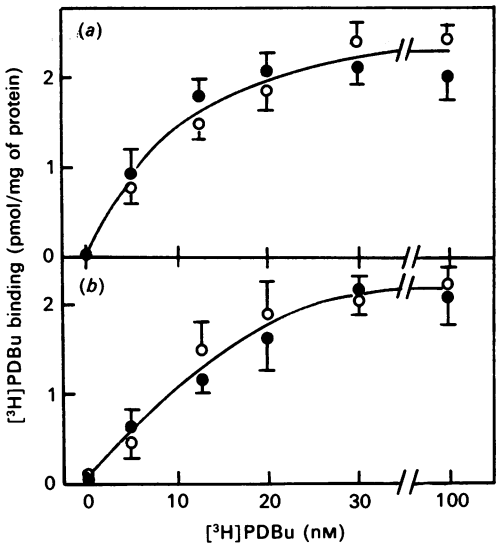

Fig. 3. [^{3}H]PDBu-binding distribution between the soluble and particulate fractions from control or 100 nM-VP-treated hepatocytes

Cells (0.9–1.0 g) were incubated for 2 min in the absence (○) or presence (●) of the hormone and immediately homogenized and centrifuged (105000 *g* for 30 min). The [^{3}H]PDBu binding was measured in the cytosol (*a*) and in the particulate fraction (*b*). Results are means ± S.E.M. of three assays.

Moreover, the PKC activity present in the particulate fraction was minimally, but significantly, decreased after the incubation with VP (36% of the total activity in the particulate fraction after 12 min of incubation; $P < 0.001$).

To determine whether larger concentrations of VP (up to 1 μM) may influence the subcellular distribution of PKC, the

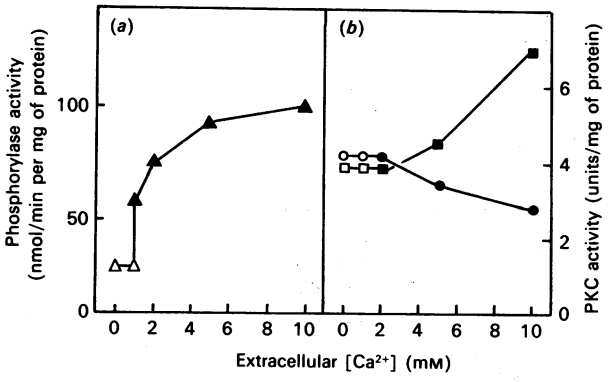

Fig. 4. Effect of Ca^{2+} on the activity of glycogen phosphorylase *a* and on PKC distribution in hepatocytes

Cells were incubated in the absence (△, ○, □) or in the presence (▲, ●, ■) of 2 μM-ionomycin. Samples were collected to measure glycogen phosphorylase activity (*a*) and PKC distribution between the soluble (○, ●) and particulate (□, ■) fractions (*b*). Results are means of two independent determinations.

hepatocytes were incubated for 2 min in the presence of increasing concentrations of hormone. The cells were analysed for the activity of glycogen phosphorylase, the changes in the intracellular free Ca^{2+} concentration, and PKC activity distribution. As Fig. 2 shows, even at saturating concentrations of VP, no changes in PKC distribution were observed. However, glycogen phosphorylase was maximally stimulated; the intracellular concentration of Ca^{2+} reached values of about 0.8 μM at 100 nM concentrations of the hormone. One possible explanation for this lack of redistribution of PKC by VP is the presence of non-accessible pools of PKC in the cytosol. For this reason, to maintain a positive control over the distribution of PKC among the soluble and particulate fractions, 100 ng of PDBu/ml was added to an incubation in the presence of 100 nM-VP, resulting in the complete disappearance of PKC from the cytosol, after its translocation towards the membranes (results not shown). This result suggests that the cytosolic enzyme was freely accessible to the phorbol and, therefore, to the metabolites that transduce the VP-binding signal.

The distribution of PKC shown in Figs. 1 and 2 was determined by monitoring its histone kinase activity. To obtain an additional criterion for the distribution of PKC after VP incubation, the amount of enzyme present in each compartment was measured by monitoring the [^{3}H]PDBu binding to the soluble and particulate fractions. Fig. 3 shows the distribution of binding sites in hepatocytes incubated in the absence or presence of 100 nM-VP for 2 min. The amounts of binding sites in the particulate and soluble fractions were 2.1–2.4 pmol/mg of tissue protein for control and VP-treated cells. The same distribution was also observed in cells incubated for 10 min with the hormone (results not shown).

As a further analysis of the factors relevant to the distribution of PKC in the hepatocytes, the concentrations of Ca^{2+} and DAG, the two signals that mediate the action of the hormone, were modulated by the use of the Ca^{2+} ionophore ionomycin, which allows the determination of the hepatocyte Ca^{2+} entry by fura-2 analysis, and the permeant DAG derivatives dioctanoylglycerol and OAG. Fig. 4 shows the activities of glycogen phosphorylase and PKC in hepatocytes incubated for 1 min with 2 μM-ionomycin and different extracellular Ca^{2+} concentrations. In the absence of ionophore, the activity of glycogen phosphorylase and the PKC distribution remained unchanged regardless of the extracellular concentration of Ca^{2+} (up to 5 mM; result not

Table 1. Ionomycin-induced redistribution of PKC in isolated rat hepatocytes

Cells were incubated for 10 min with Krebs–Ringer bicarbonate containing 1.5 mM-$CaCl_2$ and 10 μg of leupeptin/ml, and exposed to 2 μM-ionomycin. Samples were collected to analyse PKC distribution between the soluble and particulate fractions. In a parallel incubation the hepatocytes were loaded with fura-2 (5 μM) and analysed for the changes in Ca^{2+} after exposure to ionomycin. Results are means ± S.E.M. of four different experiments for PKC distribution, or means of two experiments for Ca^{2+} concentration. Values that are significantly different from the control are indicated by * $P < 0.05$ or ** $P < 0.001$.

Incubation time (min)	PKC activity (units/mg of protein)		$[Ca^{2+}]$ (μM)
	Soluble	Particulate	
0	3.5±0.2	3.1±0.2	0.17
0.5	3.4±0.3	3.3±0.1	0.92
1.0	2.9±0.2*	3.6±0.3*	1.93
2.0	2.1±0.3**	4.3±0.3**	3.73

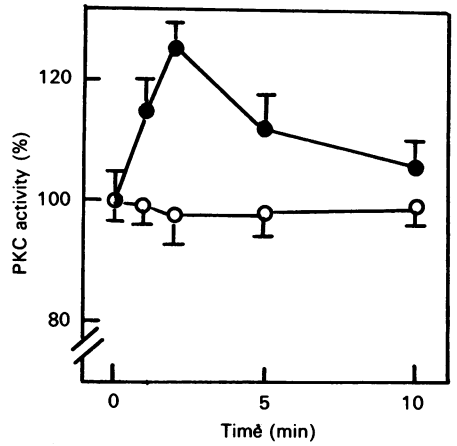

Fig. 5. Effect of OAG on the subcellular distribution of PKC in isolated hepatocytes

Cells were exposed to 250 μg of OAG/ml and samples were collected to analyse the content of PKC in the particulate fractions. ○, Control cells; ●, OAG-exposed cells. Results are means ± S.E.M. of three different experiments.

shown). However, in the presence of the ionophore, glycogen phosphorylase was activated by the entry of extracellular Ca^{2+} in a concentration-dependent form, suggesting a linear entry of Ca^{2+}. When the distribution of PKC was analysed in the same experiment, a lag in the response to the translocation towards the membranes was observed at concentrations of Ca^{2+} up to 2 mM. The changes in PKC distribution required at least 5 mM-Ca^{2+}, whereas for the activation of glycogen phosphorylase the threshold was 1 mM. When the hepatocytes were incubated in a medium containing 10 mM-Ca^{2+}, the ionophore-mediated entry of Ca^{2+} translocated about 70% of the total enzyme. These results are in agreement with previous data concerning the role of Ca^{2+} in the distribution of PKC [35].

To establish the threshold value of Ca^{2+} required to promote PKC redistribution, hepatocytes were exposed to ionomycin (2 μM) and samples were collected at several times to analyse the PKC content in the cytosol and in the particulate fractions. In parallel, hepatocytes were loaded with fura-2 and analysed for Ca^{2+} mobilization under similar conditions to those used for PKC analysis. As Table 1 shows, only concentrations of Ca^{2+} higher than 2 μM promoted significant changes in PKC distribution. These results are in agreement with the absence of PKC changes after VP exposure of the hepatocytes, since the Ca^{2+} increase elicited by this hormone is below 1 μM.

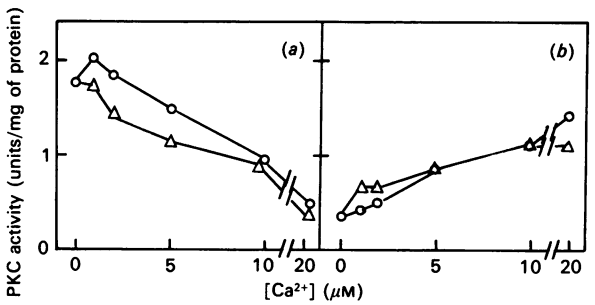

Fig. 6. Effect of Ca^{2+} and DAG on the distribution of PKC in a reconstituted system containing hepatic plasma membranes and purified soluble PKC

Plasma membranes were purified by centrifugation on sucrose gradients, and incubated for 5 min with the soluble enzyme and increasing concentrations of Ca^{2+} and in the absence (○) or presence (△) of 40 μg of OAG/ml. (*a*) PKC activity in the cytosol; (*b*) PKC activity extracted from the membranes. The incubation was stopped by centrifugation at 41000 *g* for 15 min. Proteolysis was prevented by adding 10 μg of leupeptin/ml to the assay. The reagents were treated with Chelex resin to remove any contaminant Ca^{2+}. Results are means of duplicate translocation assays *in vitro*.

To determine whether DAG may affect the translocation of PKC from the cytosol to the membranes, hepatocytes were incubated with the permeant DAGs OAG and dioctanoylglycerol. As Fig. 5 shows, high concentrations of OAG (250 μM) produced a small (20–30%) but significant time-dependent translocation of the enzyme to the membranes. The process was very rapid (less than 1 min) and remained at least for 5 min. Lower concentrations of OAG (20–100 μM) failed to redistribute PKC during the observation time. In addition to OAG, dioctanoylglycerol produced roughly similar results. When a parallel experiment was conducted, but with hepatocytes incubated for 2 min with 250 μM-OAG and 100 nM-VP, to provide a physiologically high Ca^{2+} concentration, the PKC translocation remained unchanged, suggesting a lack of synergism between both activators.

The ability of Ca^{2+} and DAG to redistribute hepatic PKC was also analysed in a cell-free system by using purified plasma membranes and hepatic PKC partially purified by DE52 chromatography. As Fig. 6 shows, Ca^{2+} at concentrations higher than 2 μM effectively promoted the translocation of the enzyme towards the membranes. The presence of leupeptin in the translocation assay was critical to obtain a quantitative recovery of PKC in the plasma membrane fractions. When OAG (10 μg/ml) was also present in the translocation assay, the PKC distribution was only slightly affected at low concentrations of Ca^{2+} (below 5 μM), which also suggests a lack of co-operativity between these PKC activators in the control of the translocation of this kinase in liver.

DISCUSSION

Translocation of PKC from the cytosol to particulate cellular compartments is a process elicited by several PKC ligands, among which are Ca^{2+}, DAG, long-chain unsaturated fatty acids and lipid moieties from lipopolysaccharides [35–37]. The extent of the translocation may vary, depending on the cell type and on the pathway used to produce these PKC ligands.

In the present work the relationship between the changes in the

concentrations of DAG or free cytosolic Ca^{2+} and their effect on the subcellular distribution of PKC were investigated in isolated hepatocytes. VP is a good agonist to produce phosphoinositide-derived metabolites, because hepatocytes contain a large number of VP receptors, higher than those for angiotensin II or related Ca^{2+}-mobilizing hormones [38,39]. Furthermore, at saturating concentrations VP preferentially mobilizes the Ca^{2+} coming from intracellular stores. Finally, the rise in the concentration of DAG elicited by VP seems to be quite stable, as determined by different groups, and it may be estimated to be approx. 2–3 nmol of fatty acid/mg dry wt. of hepatocytes [38–40]. This rise occurs despite the presence of an important DAG kinase activity, which is also stimulated by VP, that may remove part of the DAG formed [41].

In agreement with these changes elicited by VP, a VP-dependent translocation of PKC should be found from the cytosol towards the particulate compartment as a consequence of the VP-induced enhancement of Ca^{2+} and DAG. Surprisingly, VP failed to redistribute PKC in hepatocytes. This failure may not be attributed to a lack of response to the hormone, since large increases in Ca^{2+} and $InsP_3$ and in the activity of glycogen phosphorylase *a* had been measured as controls in the same experiment. According to our results and to those from other groups, phosphorylase activation is completed within 30 s [33,42]. Thus a nearly similar time course for the activation of PKC should be expected, since Ca^{2+} and DAG were produced at similar rates and the concentration of the agonist was saturating.

It has been suggested that only the membrane-bound enzyme is active after formation of a complex with DAG, Ca^{2+} and membrane phospholipids [43–45]. For example, this is the case in *ras*-transformed cells that contain high constitutive levels of DAG and where PKC is mostly found in the membrane-bound compartment. Moreover, the total content in PKC in *ras*-transformed rat fibroblasts is markedly lower than that found for control cells, suggesting that continuous activation of PKC may produce a partial degradation of the enzyme [46–48]. The same holds true for the interleukin-2-induced association of PKC with the plasma membrane in murine T lymphocytes [49].

In liver, different lines of evidence have shown that PKC is involved in the mechanism of action of VP [50,51], and therefore, according to our results, it may be suggested that only the pre-existing enzyme bound to the membranes is activated by the second messengers elicited after hormone binding. The ancillary conclusion to this result is that the soluble enzyme is not recruited to mediate the response to VP stimulation of the hepatocytes. Thus the conclusion from these results is that the concentrations of messengers required to translocate PKC from the cytosol to the membranes are higher than those produced by the hormone. Other alternatives, such as the saturation of the membrane PKC-binding capacity, or the presence of a soluble PKC pool not accessible to these messengers generated by the hormone, seem unlikely, since PDBu is able to translocate soluble PKC to the membrane completely, as occurred when increasing concentrations of Ca^{2+} and DAG were used.

A clear result obtained from these experiments is the quantification of the concentrations of Ca^{2+} and DAG, the physiological metabolites that translocate PKC, required to mobilize the enzyme to the membranes. By using a Ca^{2+} ionophore and a permeant derivative of DAG, it was possible to establish the minimal concentration of these molecules required to promote changes in PKC distribution. For OAG, we have shown that concentrations below 200 μM were unable to promote the translocation of PKC. This concentration of OAG is much higher than those required to produce activation of PKC *in vivo* [52]. For Ca^{2+}, the threshold concentration for the mobilization of PKC is about 2 μM-Ca^{2+}.

The messengers produced after VP-receptor occupancy only activate the membrane-bound enzyme, since DAG is located only in that compartment. Thus the question emerging from these results is whether to assess a biological role with respect to the distribution of PKC in hepatocytes. Our hypothesis is that the soluble enzyme constitutes a reservoir of functional enzyme that may be mobilized to the membranes to replace the particulate PKC that is proteolysed as a result of either long-sustained high concentrations of DAG or an eventual enhancement of the Ca^{2+} uptake similar to that elicited by several xenobiotics. In agreement with this suggestion is the observation that the down-regulation of PKC is mainly due to the proteolysis of the enzyme [53].

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REFERENCES

1. Majerus, P. W., Connolly, T. M., Bansal, V. S., Inhorn, R. C., Ross, T. S. & Lips, D. L. (1988) J. Biol. Chem. **263**, 3051–3054
2. Casey, P. J. & Gilman, A. G. (1988) J. Biol. Chem. **263**, 2577–2580
3. Cockcroft, S. (1987) Trends Biochem. Sci. **12**, 75–78
4. Melin, P.-M., Sundler, R. & Jergil, B. (1985) FEBS Lett. **198**, 85–88
5. Uhling, R. J., Prpic, V., Jiang, H. & Exton, J. H. (1986) J. Biol. Chem. **261**, 2140–2146
6. Taylor, J. S. & Exton, J. H. (1987) Biochem. J. **248**, 791–799
7. Wallace, M. A. & Fain, J. N. (1985) J. Biol. Chem. **260**, 9527–9530
8. Berridge, M. J. (1984) Biochem. J. **220**, 345–360
9. Berridge, M. J. & Irvine, R. F. (1989) Nature (London) **341**, 197–205
10. Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. & Nishizuka, Y. (1980) J. Biol. Chem. **255**, 2271–2276
11. Kikkawa, U., Kishimoto, A. & Nishizuka, Y. (1989) Annu. Rev. Biochem. **58**, 31–44
12. Nishizuka, Y. (1986) Science **233**, 305–312
13. Nishizuka, Y. (1988) Nature (London) **334**, 661–665
14. Bosca, L.,Rousseau, G. G. & Hue, L. (1985). Proc. Natl. Acad. Sci. U.S.A. **82**, 6440–6444
15. Ballester, R., Furth, M. E. & Rosen, O. M. (1987) J. Biol. Chem. **202**, 2688–2695
16. Wolfman, A. & Macara, I. G. (1987) Nature (London) **325**, 359–361
17. Bollag, G. E., Roth, R. A., Beaudoin, J., Mochly-Rosen, D. & Koshland, D. E., Jr. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 5822–5824
18. Kraft, A. S., Anderson, W. B., Cooper, H. L. & Sando, J. J. (1982) J. Biol. Chem. **257**, 13193–13196
19. Copalakrishna, R., Barsky, S. H., Thomas, T. P. & Anderson, W. B. (1986) J. Biol. Chem. **261**, 16438–16445
20. Anwyl, R. (1989) Trends Pharmacol. Sci. **10**, 236–239
21. Hue, L., Felíu, J. E. & Hers, H. G. (1978) Biochem. J. **176**, 791–797
22. Palmer, S., Hughes, K. T., Lee, D. Y. & Wakelam, M. J. O. (1988) Cell. Signalling **1**, 147–156
23. Palmer, S., Hughes, K. T., Lee, D. Y. & Wakelam, M. J. O. (1988) Biochem. Soc. Trans. **16**, 991–992
24. Cobbold, P. H. & Rink, T. J. (1987) Biochem. J. **248**, 313–328
25. Stalmans, W. & Hers, H. G. (1975) Eur. J. Biochem. **54**, 341–350
26. Hucho, F. & Krüger, M. (1987) FEBS Lett. **211**, 207–210
27. Díaz-Guerra, M. J. M., Sanchez-Prieto, J., Bosca, L., Pocock, J., Barrie, A. & Nicholls, D. (1988) Biochim. Biophys. Acta **970**, 157–165
28. Bosca, L., Díaz-Guerra, M. J. M. & Mojena, M. (1989) Biochem. Biophys. Res. Commun. **160**, 1243–1249
29. Wolf, M., Le Vine, H., III, May, W. S., Jr., Cuatrecasas, P. & Sahyoun, N. (1985) Nature (London) **317**, 546–549
30. Moruzzi, M., Barbiroli, B., Monti, M. G., Tadolini, B., Hakim, G. & Mezzetti, G. (1987) Biochem. J. **247**, 175–180
31. Emmelot, P., Bos, C. J., van Hoeven, R. P. & Blitterwijk, W. J. (1974) Methods Enzymol. **31**, 75–90
32. Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
33. Thomas, A. P., Alexander, J. & Williamson, J. R. (1984) J. Biol. Chem. **259**, 5574–5584

34. Creba, J. A., Downes, C. P., Hawkins, P. T., Brewster, G., Michell, R. H. & Kirk, C. J. (1983) Biochem. J. **212**, 733–747
35. Phillips, W. A., Fujiki, T., Rossi, M.,Korchak, H.M. & Johnson, R. B., Jr. (1989) J. Biol. Chem. **264**, 8361–8365
36. Murakami, K. & Routtenberg, A. (1985) FEBS Lett. **192**, 189–193
37. Wightman, P. D. & Raetz, C. R. H. (1984) J. Biol. Chem. **259**, 10048–10052
38. Bocckino, S. B., Blackmore, P. F. & Exton, J. H. (1985) J. Biol. Chem. **260**, 14201–14207
39. Lynch, C. J., Blackmore, P. F., Charest, R. & Exton, J. H. (1985) Mol. Pharmacol. **28**, 93–99
40. Rodriguez de Turco, E. B. & Spitzer, J. A. (1988) Biochem. J. **253**, 73–79
41. Rider, M. H. & Baquet, A. (1988) Biochem. J. **255**, 923–928
42. Thomas, A. P., Marks, J. S., Coll, K. E. & Williamson, J. R. (1983) J. Biol. Chem. **258**, 5716–5725
43. Bell, R. M. (1986) Cell **45**, 631–632
44. Kraft, A. S., Anderson, W. B., Cooper, H. L. & Sando, J. J. (1982) J. Biol. Chem. **257**, 13193–13196
45. Woodgett, J. R., Hunter, T. & Gould, K. L. (1987) in Cell Membranes; Methods and Reviews (Elson, E. L., Frazier, W. A. & Glaser, L., eds.), vol. 3, pp. 215–340, Plenum Press, New York
46. Lacal, J. C., Fleming, T. P., Warren, B. S., Blumberg, P. M. & Aaronson, S. A. (1987) Mol. Cell. Biol. **7**, 4146–4149
47. Price, B. D., Morris, J. D. H., Marshall, C. J. & Hall, A. (1989) J. Biol. Chem. **264**, 16638–16643
48. Huang, M., Childa, K., Kamata, N., Nose, K., Kato, M., Homma, Y., Takenawa, T. & Kuroki, T. (1988) J. Biol. Chem. **263**, 17975–17980
49. Farrar, W. L. & Anderson, W. B. (1985) Nature (London) **315**, 233–235
50. Garrison, J. C., Johnsen, D. E. & Campanile, C. P. (1984) J. Biol. Chem. **259**, 3283–3292
51. García-Sainz, J. A. & Hernandez-Sotomayor, S. M. T. (1987) Eur. J. Biochem. **163**, 417–421
52. Rozengurt, E., Rodriguez-Peña, A., Coombs, M. & Sinneth-Smith, J. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 5748–5752
53. Young, S., Parker, P. J., Ullrich, A. & Stabel, S. (1987) Biochem. J. **244**, 775–779

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